Available online at www.sciencedirect.com
ScienceDirect
Environmental Pollution 144 (2006) 453–462
ENVIRONMENTAL POLLUTION
www.elsevier.com/locate/envpol

Review

Use of the genus *Artemia* in ecotoxicity testing

Bruno S. Nunes [a,b,*], Félix D. Carvalho [c], Lúcia M. Guilhermino [a,b], Gilbert Van Stappen [d]

[a] *Instituto de Ciências Biomédicas de Abel Salazar, Departamento de Estudos de Populações, Laboratório de Ecotoxicologia, Universidade do Porto, Largo Prof. Abel Salazar 2, 4099-003 Porto, Portugal*
[b] *Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4150-123 Porto, Portugal*
[c] *REQUIMTE, Departamento de Toxicologia da Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[d] *Laboratory of Aquaculture and Artemia Reference Centre, Ghent University, Rozier 44, B-9000 Ghent, Belgium*

Received 11 November 2004; accepted 29 December 2005

The physiology, reproductive processes and general use of Artemia in modern ecotoxicological testing are reviewed.

Abstract

Information related to varied uses of several species of the genus *Artemia* (commonly known as brine shrimp), is dispersed among literature from several scientific areas, such as Ecology, Physiology, Ecotoxicology, Aquaculture and Genetics. The present paper reviews information related to *Artemia* that may be considered relevant for ecotoxicity testing. Integration of different areas of scientific knowledge concerning biology, life cycle and environmental needs of *Artemia* is of crucial importance when considering the interpretation of results drawn from tests involving this genus. Furthermore, this paper provides suggestions to overcome problems related to toxicity assessment with the use of *Artemia* as test organism in bioassays, under the scope of estuarine, marine and hypersaline environments. Aspects related to variability in results, adoptable toxicity end-points, culture conditions, characteristics of species and strains, influence of geographical origins over physiological features and responses to exposure to chemical agents are considered.

Keywords: *Artemia*; Culture; Cysts; Bioassays; Ecotoxicology

1. Introduction

Several areas of scientific knowledge disperse information related to the genus *Artemia* (brine shrimp). An integration of this information is important for toxicologists working with this genus. *Artemia* is subdivided into six generally recognized bisexual species and a large number of parthenogenetic populations, is characterized by common features such as adaptability to wide ranges of salinity (5–250 g L^{-1}) and temperature (6–35 °C), short life cycle, high adaptability to adverse environmental conditions, high fecundity, bisexual/parthenogenetic reproduction strategy (with nauplii or cysts production), small body size, and adaptability to varied nutrient resources as it is a non-selective filter feeder. Per se, the intrinsic features of this genus turn it into a suitable organism for use in Ecotoxicology, guaranteeing reliability, feasibility and cost-effectiveness in routine and/or research ecotoxicity practices. The present paper intends to highlight advantages, characteristics and general physiological features of the distinct species of *Artemia*, regarding the use of these organisms in Ecotoxicology. Generally, several criticisms against the use of *Artemia* have been presented in spite of its current practical use. Persoone and Wells (1987) summarized in a remarkable way the standpoint of criticism against the inclusion of *Artemia* as test organism in standardized bioassays: the absence of *Artemia* in most of the marine ecosystems; its presumed lack of sensitivity to chemical exposure due to the intrinsic

* Corresponding author. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Rua Carlos da Maia, 296, 4200-150 Porto, Portugal. Tel.: +225074630; fax: +225074637.
E-mail address: bruno@ufp.pt (B.S. Nunes).

0269-7491/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.envpol.2005.12.037

resistance to extreme salinity conditions; and the alleged unsuccessful works of some researchers using *Artemia* in their own experiments. However, a great number of characteristics that turn this organism into a suitable species for use in Ecotoxicology may also be pointed out, including a wide geographic distribution, relatively simple laboratory culture and maintenance, resistance to manipulation, short life-cycle, large offspring production and the existence of a considerable amount of information about some species.

2. General features of the genus *Artemia*

Artemia has a wide geographical distribution. The species of this genus possess an uncommon adaptability to extreme conditions, thus being found in environments where other life forms are not sustainable (Triantaphyllidis et al., 1998). The different species of the genus *Artemia* present one common characteristic, that is, their strong adaptability to hypersaline environments, such as permanent salt lakes, coastal lagoons and man-made salt pans where evaporation of seawater results in high sodium chloride concentrations. High salt concentrations can also be found in other habitats colonized by *Artemia*, such as temporal salt lakes, subjected to unpredictable floods. The habitats in which the genus *Artemia* is found are characterized by the absence of predatory animal species. Therefore, in such environments, the evolution of *Artemia* populations is favoured by the abundance of bacteria, protozoa and algae that are the basis of the *Artemia* diet (Amat, 1985).

Several studies have been performed in order to take advantage of the high adaptation patterns of *Artemia* concerning nutrient resources. For instance, *Artemia* is able to use several nutrient resources, such as wheat bran, soybean meal, rice bran and whey powder (Dobbeleir et al., 1980; Sorgeloos et al., 1980). Other nutrient sources suitable for *Artemia* production are fishpond effluents (Zmora et al., 2002) or inert commercial diets, such as Nestum® (Naegel, 1999). In spite of this high adaptability to varied nutrient resources, the concentration of nutrient supplies is highly relevant for *Artemia*, as shown by the works of Evjemo and Olsen (1999), which clearly established a relation between growth and total food intake under controlled abiotic conditions (growth and ingestion rate were positively correlated with nutrient concentrations).

Tolerance to extremely variable oxygen concentrations is another common trait to several species of *Artemia*, as stated by Amat (1985), which allows specimens to successfully face environmental adversities under extreme conditions.

The wide distribution of *Artemia* species and strains can also pose several concerns related to the site where cysts and specimens can be collected. The provenience of *Artemia* cysts is a most important factor for every of its posterior uses, because different strains with distinct patterns of growth, survival and reproduction occur at distinct sites. On the other hand, in the same natural *Artemia* samples, cysts of several species of the genus *Artemia* may be found. Triantaphyllidis et al. (1994) suggested a simple procedure, which allows the detection of a mixed nature in *Artemia* commercial cysts samples, through their incubation at high temperatures. According to this technique, the incubation of cysts at temperatures between 35 and 36 °C permits the hatching of cysts from the bisexual species *A. franciscana* but not that of parthenogenetic species. Therefore, the presence of species with different reproduction strategies may be detected. Furthermore, according to Abatzopoulos et al. (1997), *Artemia* species can be distinguished, through a new method based on the protein characteristics of the cyst's membrane. Through restriction fragment length polymorphism (RFLP) analysis of a 1500 bp mitochondrial rDNA fragment, Bossier et al. (2004) worked out a methodology to authenticate cyst samples down to the species level.

Hatching characteristics can vary according to the geographic origin of cysts (Vanhaecke and Sorgeloos, 1982; Vanhaecke and Sorgeloos, 1983) and according to conditions of harvesting, processing and storage of a particular batch of cysts (Lavens and Sorgeloos, 1987, 1996). Geographic origin of cysts may also influence several measurable parameters in neonates, as described by Vanhaecke and Sorgeloos (1980a): volume and diameter of hydrated, untreated and decapsulated cysts; chorion thickness; length, dry weight, ash free weight and volume index of freshly hatched nauplii. Origin of strains can have consequences on the growth, survival and reproduction of *Artemia* specimens in function of abiotic factors (e.g. salinity and temperature), as shown by Vanhaecke and Sorgeloos (1980b) and Triantaphyllidis et al. (1995). The complete knowledge and control of the origin of cysts is highly important for research.

A considerable scientific investment has been made in order to study the taxonomic relationship between *Artemia* species and strains, according to their geographic origin, taking into consideration morphological, reproductive, physiological, morphometrical, biochemical and genetic features. Subdivision of the genus into species, the occurrence of (diploid and polyploid) parthenogenetic and of bisexual strains, and generally the entire problem of genetic flexibility and speciation have very direct implications in the field of aquaculture (Gajardo et al., 2002): the quality of the *Artemia* product differs from strain to strain and from location to location, e.g. in terms of hatching and biometrical characteristics, as a result of genotypic and phenotypic variation. Also the nutritional value of *Artemia*, especially for marine organisms, is not constant but varies among strains and within batches of each strain, causing unreliable outputs in marine larviculture (Léger et al., 1986, 1987a). Through multidisciplinary studies in the 1980s both the causes for the nutritional variability in *Artemia* and the methods to improve poor-quality *Artemia* were identified. To optimise the use of the *Artemia* stocks in the market, and to diversify the use of natural resources, a variety of different research initiatives were launched, leading to improved techniques for cyst harvesting, processing and storing, and for nauplii applications, and exploitation of new natural resources (Lavens and Sorgeloos, 1996; Van Stappen, 2003). In addition, numerous managed ponds and saltworks worldwide started providing small quantities of good quality cysts (1–20 metric tons each), providing interesting opportunities for local commercial development (Lavens and Sorgeloos, 2000; Dhont and Sorgeloos, 2002).

After harvesting and processing, cysts are available in cans as storable 'on demand' live feed. Upon a few hours of

incubation in seawater, the free-swimming nauplii can directly be fed as a nutritious live food source to the larvae of a variety of marine as well as freshwater fish and crustaceans, which makes them the most convenient, least labour-intensive live food available for aquaculture (Sorgeloos et al., 1998, 2001). By bio-encapsulating specific amounts of particulate or emulsified products rich in highly unsaturated fatty acids in the brine shrimp metanauplii, the nutritional quality of *Artemia* can be further tailored to suit the nutritional requirements of fish and shrimp larvae (Léger et al., 1987b; Lavens and Sorgeloos, 1996; Dhont and Van Stappen, 2003). Furthermore, a better knowledge of the biology of *Artemia* was at the origin of the development of other *Artemia* products, e.g. disinfected and decapsulated cysts (Bruggeman et al., 1980) and various biomass preparations, which presently have application in hatchery, nursery and broodstock rearing.

Work from authors such as Browne and Wanigasekera (2000) indicated that several other physiological major differences occur between distinct species of *Artemia*, according to environmental conditions during the development of the organisms (e.g. temperature and salinity). These differences were readily noticeable when considering parameters such as survival and reproductive performance in established combinations of the interdependent variables temperature and salinity. The distinct patterns evidenced by all species of the genus *Artemia* (in which several sexual dimorphic species and a parthenogenetic species were included) related to parameters such as 21-day survival, LT_{50}, lifespan and reproductive traits, confirmed the high intrinsic variability in physiological responses towards salinity and temperature fluctuations among strains and between species of the same genus (Vanhaecke et al., 1984; Bowen et al., 1985; Browne et al., 1988; Browne and Halanych, 1989; Vanhaecke and Sorgeloos, 1989; Triantaphyllidis et al., 1995).

3. Sexual versus parthenogenetic reproduction

During their evolution, populations tend to maximise their fitness through the increase of the proportion of individuals well adapted to the environment, while assuring the capacity of response to environmental changes by maintaining a small proportion of less adapted individuals. In this process, reproduction plays a determinant role. Therefore, species of the genus *Artemia* adopted different reproductive strategies allowing the minimisation of the ''costs'' required to assure the capability of the population response at long term and also assumed distinct strategies to overcome permanent and/or periodical adverse conditions of the habitats in which they live.

Reproduction of *Artemia* species can be performed under two major modalities: sexual or parthenogenetic. In the Old World (Europe, Africa and Asia) both parthenogenetic and sexual (natural) populations occur, whereas in the New World (Americas) only sexual strains are found (Browne, 1992; Van Stappen, 2002). Coexistence of more than one strain has been recorded for a number of sites; in most of these cases a parthenogenetic and a sexual strain share the same habitat. However, this coexistence is often just partially overlapping in space and/or time: due to the different tolerance and preference of each strain for abiotic conditions, predominant occurrence of each of the coexisting strain is linked to a certain season and/or niche within the habitat (reviewed by Van Stappen, 2002). Temperature is a crucial parameter for the establishment of different species in a given area: experiments conducted in order to assess the effects of temperature over the relative success and competition of parthenogenetic vs. sexual species indicated that, at temperatures of 25 °C, parthenogenetic species systematically eliminated the sexual species (Browne and Halanych, 1989) due to higher offspring production. Temperatures about 15 °C are favourable to sexual species, but at higher temperatures (approximately 30 °C), death occurred prior to sexual maturation for sexual species (Barata et al., 1996). These data are of great importance when considering the occurrence of inter- and intra population fluctuations in many ecosystems, associated with changes in average temperature along the year. Nevertheless, significant variations exist in the response of different populations (even belonging to the same species) towards abiotic conditions, and often precise predictions can only be made at the strain level.

An additional importance can be added to reproductive features among the different strains of the genus *Artemia* when considering its use as test organism in Ecotoxicology. Control of reproductive patterns is of fundamental importance and must be extensively known to fulfil the requirements of cost-effectiveness, homogeneity and predictability of responses among tests. Conditions for rearing, maintenance, maturation and development of tests must be accomplished under optimal conditions, defined for each strain, and must not allow significant variations, which may introduce variability in results. Reproduction in the genus *Artemia* can originate two types of offspring, namely *nauplii* and *cysts*. *Artemia* can produce resistant embryos (cysts), prepared to face stressful environmental conditions (e.g. increase in salinity with eventual desiccation and lowered temperatures). During this period of encystment, designated as diapause or cryptobiosis, and characterized by the absence of noticeable metabolic activity (Clegg, 2001), the structural integrity and the viability of the embryo can be maintained for several years, especially if stored under suitable conditions (absence of oxygen and low temperature). The ability to produce cryptobiotic cysts is an adaptation of the organism to overcome natural adverse conditions. Under optimal conditions, embryos go through normal development in the ''uterus'' and are spawned under the form of nauplii, a self-sustainable, independent, free-swimming organism. The production of cysts or the release of full-developed nauplii is triggered by environmental signals, resulting in a strain-dependent response. As a general rule, however, females switch from nauplii to cyst production when environmental conditions turn unfavourable or unstable (Lavens and Sorgeloos, 1987).

Authors such as Browne and Wanigasekera (2000) published extensive research related to the study of simultaneous influence of temperature and salinity (two of the most important conditioners of marine and brackish organisms) over cultures of five species of *Artemia*, four sexual and one parthenogenetic species.

Results showed several combinations of salinity-temperature for which survival and reproductive success of the different species were maximal, but several combinations of species-salinity-temperature were held responsible for life-threatening challenges (13 in 45 combinations induced 100% mortality). In general terms, higher temperatures increased mortality, but the same was not true for salinity. The use of a large number of combinations allowed stating that no optimal common combination salinity-temperature for all tested species can be obtained. Every species studied by these authors has clear salinity-temperature preferences, similar to the environmental conditions found in their places of origin. The reproductive success of the parthenogenetic strain used in this work was found to be highly dependent on subtle environmental fluctuations, and only reached full reproductive maturity in few of the tested combinations of salinity and temperature, which allowed to characterize this strain as a niche specialist. A parameter strongly influenced by temperature was the length of the pre-reproductive period of females; this parameter was considerably reduced with the increase of temperature, while salinity did not influence it in a linear manner. Definition of optimal salinity-temperature conditions is a warranty of good general physiological condition: females subjected to their optimal salinity-temperature conditions spent the majority of their lives in a continuous reproductive period. The parthenogenetic strain took considerably longer periods to reach reproductive maturity when compared to the sexual strains; in spite of this delay, this *Artemia* strain has been considered as the most prolific one under those experimental conditions. Nevertheless, general considerations can be drawn from this research when attempting to establish culturing conditions for the different *Artemia* species: satisfactory reproductive output (maximum reproductive periods and number of offspring) might be accomplished when subjecting individuals to a temperature of 24 °C and at a salinity of 120 or 180 g L^{-1}. Long pre-reproductive periods are a consequence of exposure both to low temperatures (15 °C) and high temperatures (30 °C), which can also imply a sensible reduction in length of reproductive periods.

4. *Artemia* in ecotoxicology

Major attempts to use representative and autochthonous organisms in multitrophic and multispecies assessments have been made in recent years, seeking more accurate approaches and reliable extrapolations for real scenarios. Standardized laboratory procedures involve culture of animals and the maintenance of constant abiotic conditions to assure the reproducibility of results. Choice of the more adequate animal model should take into account global aspects of biology, life cycle, adaptability to laboratory conditions, ecological relevance, systematic use, and practical conditions of maintenance and sustainability of laboratory cultures. *Artemia* use in Ecotoxicology poses a reasonable number of answerable questions, namely practical considerations of laboratory culture and attainment of cyst, thus making achievable a sustainable development of *Artemia*-based bioassays. Nevertheless, great attention is needed to fully comprehend the complexity of distinct species or strains from geographical distinct sites and the possible implications of these general characteristics over culture practices and ultimate result interpretation. *Artemia* is by far one of the most striking examples of organisms well adapted to laboratory practice, as long as a strict control over laboratory procedures and methodologies is maintained. On the other hand, its use as test organism is representative of the effort to reduce the scale of test organisms, with concomitant reduction in test volumes, amount of produced waste, and space needed to perform testing protocols (Blaise, 1998).

4.1. Origin of test organisms

Full characterization of biological samples is a fundamental aspect in ecotoxicity testing. The possibility of simultaneously using several different strains of *Artemia*, due to contamination, deficient geographical localization of origin or incomplete characterization of cyst samples, can lead researchers to erroneous results. Work performed by Varó et al. (1998) showed consistent distinct patterns for chlorpyrifos toxicity according to the species, strain and even geographical origin of the cyst lot incubated for the testing purposes. Responses of distinct nature and magnitude can be found among strains as also evidenced by Browne (1980), when studying acute toxicity and reproductive traits of five strains of brine shrimp following copper sulphate exposure.

4.2. Sensitivity of Artemia-based toxicity bioassays

In spite of its massive use in toxicological testing, earlier studies refer *Artemia* as a less sensitive species for ecotoxicity studies, when compared to other test organisms under the same experimental conditions such as *Streptocephalus rubricaudatus* and *S. texanus* (Crisinel et al., 1994), *Echinometra lucunter* and *Crassostrea rhizophorae* (Nascimento et al., 2000) or algal species, such as *Selenastrum capricornutum* and *Dunaliella tertiolecta* (Gaggi et al., 1994). Guerra (2001) also reported lower sensitivity of *Artemia*-based assays, when compared to commercially available screening tests (Microtox® and Rotoxkit M®) and to the standardized test with *D. magna*. Okamura et al. (2000) also reported a diminished sensitivity of *Artemia*, when comparing the responsiveness of several organisms towards the antifouling agent Irgarol 1051. *Artemia* was considered to be the least sensitive organism among a group, which included varied species of crustaceans, such as *Daphnia magna*, *Daphnia pulex* and *Thamnocephalus platyurus*. *Artemia* was shown to be more tolerant than *Aedes taeniorhynchus* after exposure to the insecticides aldicarb, dimethoate, imidacloprid and tebufenozide (Song and Brown, 1998). Sensitivity of *Artemia* was also questioned by Nałęcz-Jawecki et al. (2003) when performing a comparative study of the sensitivity of several bioassays (*Vibrio fischeri*, *Spirostomum ambiguum* and *Tetrahymena thermophia*) to 15 quaternary ammonium compounds. *Artemia franciscana* was reported to be the least sensitive organism tested. On the other hand, researchers such as Hlywka et al. (1997) noticed similar sensitivities to the mycotoxin fumonisin B_1 when simultaneously using the acute screening toxicity assay

with *Artemia* and chicken embryo screening test, but preferred the former due to simplicity of procedures and lower volumes of toxicant required to develop the test. *Artemia* was found to be more sensitive than *Daphnia similis* and *Ceriodaphnia dubia* to niclosamide (Oliveira-Filho and Paumgartten, 2000).

4.3. End-point selection

The end-point selection for ecotoxicity testing is sometimes a decisive factor to be discussed, as the information considered vital may or may not be fully obtained when varied end-points are considered. Most frequently, cyst-based toxicity assays involving *Artemia* refer to a well-accepted end point as criterion: mortality of the recently (instar II-III stage) hatched nauplii. Besides the use of standardized development phases of *Artemia*, several studies investigated the suitability of specimens of different ages, such as the ones described by Barahona and Sánchez-Fortún (1996); this study evaluated several age classes of *Artemia salina* and the conclusions pointed to a greater relative sensitivity of the 48-hours old specimens, for the majority of the tested compounds. Studies were also performed in order to evaluate the suitability of *Artemia* cysts as test organisms (Vismara, 1998), prior to hatching and without incubation. Viability (hatchability) of cysts after exposure was selected as end-point criterion, and comparisons between the two approaches (mortality of nauplii and hatchability of cysts) were made to assess differences in sensitivity for both tests (Carballo et al., 2002). This study indicated high concordance between results, and considered useful the complementary development of the two assays.

Behavioural parameters were also suggested to serve as end-point for ecotoxicity assays. Di Delupis and Rotondo (1988) proposed the use of the phototactic response of *Artemia* nauplii as a suitable response to environmental stressing compounds.

Artemia can also be used in tests based on biomarkers, such as enzymatic activity. The work of Varó and colleagues (Varó et al., 2002a,b) presented a test based on cholinesterase inhibition with two species of the genus *Artemia* (namely *A. salina* and *A. parthenogenetica*) for the assessment of effects due to exposure to organophosphorous pesticides (dichlorvos and chlorpyrifos).

5. Reducing variability as a fundamental requirement for ecotoxicity testing

Diverse factors can alter the reliability of results in ecotoxicity testing: environmental conditions, related to non-genetic factors (temperature, pH and chemical composition of the medium, oxygen, photoperiod, nutrients, diseases, population effects) and genetic characteristics of the organisms used in tests (Soares et al., 1992). These sources of variation may be minimised by a convenient understanding of concepts underlying the use of animals for research purposes: the first source of variation is inherently dependent on maintenance/test conditions, and can be reduced through a standardization of both culture and test protocols. The second source is strictly dependent on the animal species used, its geographical provenience, and its strain and life cycle characteristics. Natural variability of organisms can be theoretically surpassed through the use of genetically similar individuals, held under strict control and artificial abiotic conditions. This state of cautious controlled environment can end in the maximum similarity: a clone. The use of *Artemia* clones, whenever a parthenogenetic population is selected to serve as test-organism, might take into account an exhaustive characterization of the clone. Parthenogenetic *Artemia* exhibits a high variability according to populations and to geographical localizations, in terms of ecological background, ploidy level, life history traits and genetic characteristics, such as chromosomal, allozymic and mitochondrial DNA. The general distinctions among parthenogenetic strains of *Artemia* may lead to interclonal variations of response to stressing factors and these variations of response are similar to interfamilial variation occurring among distinct families of sexual strains, as stated by Browne et al. (2002). According to the same study, environmental components of variation systematically surpassed variation due to genetic components, thus increasing the importance earlier attributed to a careful control of environmental variables in laboratory culture of *Artemia*. However, care must be taken to avoid uncertainty related to genetic differences between specimens, and environmental conditions must be optimised and kept stable to avoid further needs of adaptive genetic recombinations.

The majority of work performed so far involving *Artemia* as test organism in Ecotoxicology is related to the use of cysts (Migliore et al., 1993, 1997) and cyst-hatched nauplii of *Artemia*, Artoxkit® (Vanhaecke et al., 1980a,b, 1981a,b; Persoone and Vanhaecke, 1981; Vanhaecke and Persoone, 1981; Persoone and Wells, 1987). Cysts are produced both in bisexual and asexual (parthenogenetic) reproduction under stressing environmental conditions. In the genus *Artemia* there is no cyclic bisexual versus parthenogenetic mechanism like in rotifers and cladocerans. Therefore, cyst production may reflect the occurrence of genetic variation in order to permit higher adaptability to new and adverse environmental conditions; however, genetic variability is not desired in most of ecotoxicity assays since it may be an important source of variability in results. Nevertheless, the use of cysts in Ecotoxicology is highly advantageous, whenever short-term bioassays are routinely demanded, for early toxicity screening. Other major advantages are ascertained availability of test organisms, the low cost and simplicity in use.

Artemia is commercially available in the form of cysts, small spherical-like structures of high physical and chemical resistance, specialized in subsisting under extremely adverse conditions. *Artemia* cysts are available from varied sources, according to their geographical site of origin. The concept of reference cyst was introduced in the early eighties by Sorgeloos (1980), by analysing several characteristics of cysts (hatching, biometrics, fatty-acid content, pesticide contamination and effectiveness as larval food for various crustaceans and fishes). Persoone and Wells (1987) described a Round Robin (Intercalibration) exercise involving the use of reference cysts of *Artemia* in ecotoxicity assays, aiming at the determination of parameters such as reliability, accuracy and precision of this standardized methodology. This test protocol was then

subjected to discussion and validated for routine, screening toxicology tests. Conclusions drawn from years of scientific work, sustained by debate over *Artemia* characteristics as test organism, supported the attainment of a commercially available, off-the-shelf ecotoxicity test, named ARTOXKIT® (MicroBioTests Inc., Nazareth, Belgium).

6. Major evolutionary perspectives in ecotoxicology testing using *Artemia*

The early 1980s witnessed large developments in Ecotoxicology as an autonomous science in modern scientific world. New concepts were brought to actual research, as a part of an effort to adapt cost-effective procedures, which comply with accuracy, reliability and ecological relevance. These trends intend to extrapolate from laboratory results to holistic visions of ecosystems. An increasing number of animal species was adapted to laboratory culture conditions. These concepts served as a basis for the development of new methods, such as the already mentioned cyst-based screening toxicity assays (Vanhaecke et al., 1980a,b, 1981a,b; Persoone and Vanhaecke, 1981; Vanhaecke and Persoone, 1981; Persoone and Wells, 1987), which represent highly valuable answers to general laboratory needs, due to their general availability and easy manipulation. An international intercalibration exercise, described by Persoone et al. (1992), stated that 87% of all the involved participants were able to successfully complete the standardized test with *Artemia*, and evaluated the test results as simple to interpretation, reliable and accurate. Nevertheless, the assumed end-point for the mentioned cyst-based assays (mortality, defined as total immobilization after a period of 10 s) was not always well interpreted, due to the assumption of ''mortality'' for those animals that sunk in the testing medium but still exhibited appendages movements. Other parameters can be considered as reliable end-points, such as growth of *Artemia* individuals, described by Sarabia et al. (1998a).

Progress regarding the use of *Artemia* in Ecotoxicology was also made in such areas as development of diets for test-organisms used in toxicological studies, improvement of culture-related methods, description of acute or short-term toxicity procedures, comparative studies on sensitivity, reports on factors affecting reproducibility of results, comparisons of sensitivity among different test organisms, evaluation of toxicity for several classes of compounds, performance of Quantitative Structure-Activity Relationship (QSAR) studies, biomarkers applicability, reproductive and developmental assays, impact on food chains and modelling of ecosystems.

The present status of Ecotoxicology shows a thorough use of short-term acute cyst-based toxicity screening assays. Use of cysts is highly advantageous whenever needs concerning screening toxicity tests arise. In the past, research has focused on the use of *Artemia* nauplii as test organism for a wide variety of contaminants, such as metals (Sarabia et al., 1998b; Hadjispyrou et al., 2001; Brix et al., 2003), trace elements (Petrucci et al., 1995), s-triazine herbicides (Varó et al., 2002a,b), acrylonitrile (Tong et al., 1996), carbamates (Barahona and Sánchez-Fortún, 1998), toxic cyanobacteria (Vezie et al., 1996; Beattie et al., 2003), endosulfan (Varó et al., 1997), pollutants produced by incineration plants (Knulst and Sodergren, 1994), antifouling compounds (Okamura et al., 2000; Hadjispyrou et al., 2001), pharmaceuticals (Touraki et al., 1999; Parra et al., 2001), organophosphorous insecticides (Sánchez-Fortún et al., 1996), antifouling agents (Panagoula et al., 2002), mycotoxins (Panigrahi, 1993; Hlywka et al., 1997) and organic solvents (Barahona-Gomariz et al., 1994). *Artemia* nauplii have also been used in marine toxicity testing in coastal areas (Wells, 1999; Nipper, 2000), and for the establishment of alternative toxicity tests (Calleja et al., 1994; Parra et al., 2001). *Artemia*-based bioassays have also been used in The Netherlands as standardized tools for toxicity screening under estuarine and marine conditions (Creasel, 1990). Guerra (2001) included *Artemia* in a battery of ecotoxicity tests to assess the toxicity of phenolic compounds in industrial effluents, and proposed it as a useful tool for posterior use under brackish water environments. Tothill and Turner (1996) also highlight the advantageous use of *Artemia*-based assays for toxicity testing in water treatment and for rapid screening of toxicity of cyanobacterial blooms. Recent advances in toxicity testing led Kapanen and Itävaara (2001) to consider the possibility of the use of *Artemia* as a suitable test organism for the assessment of toxic effects of composts (resulting from industrial waste) on ecosystems. These authors suggest that, as a direct consequence of the lack of established methods for testing composts, acute toxicity may be assessed using several methods such as the ones involving invertebrates, including the *Artemia* genus. These two authors followed some of the principles referred by Blaise (1998) when considering the growing need for toxicity testing methods involving small-sized structures and organisms, like *Artemia*.

Varó et al. (2000) considered *Artemia* as a potential bioaccumulation vector of environmental contaminants, due to its position in the food chains (primary consumer). Their study indicated that bioaccumulation of chlorpyrifos (pesticide employed in agricultural procedures) was likely to occur under laboratory conditions, and thus could be considered a potential risk for aquatic fauna. The authors stated that chlorpyrifos-contaminated *Artemia* was responsible for biomagnification phenomena, with increased levels of contamination in two fish species, namely *Gambusia affinis* and *Aphanius iberus*. Varó et al. (2002a,b) described similar results in a more recent work. Fish (*Aphanius iberus*) feeding upon chlorpyrifos-treated *Artemia* accumulated this compound, and biomagnification values showed a continuous decrease during the bioaccumulation phase. However, after pesticide treatment, fish fed with uncontaminated preys rapidly eliminated the pesticide accumulated via food. This rapid elimination ultimately conduced to undetectable levels of chlorpyrifos in tissues from treated fish. This aspect of bioaccumulation is particularly important in view of human consumption of harvested fish, usually fed (at least during a period of its life) with *Artemia* juveniles. Bioaccumulation and eventual threat posed to humans through biomagnification was analysed by Petrucci et al. (1995) when considering *Artemia* biomass collected in different regions

of Sardinia, Italy. This study was conducted in order to identify and quantify the possibility of heavy metal bioaccumulation in *Artemia*; results showed no bioaccumulation in fish preying on *Artemia*. Bioaccumulation thus seems to be ruled by toxicant-specific mechanisms, and further studies are needed to clarify the position of *Artemia* as vector of bioaccumulation and biomagnification processes. Bioaccumulation was also the issue of the study by Hadjispyrou et al. (2001), which established the levels of accumulation of organotins and heavy metals (tin, cadmium and chromium) by individuals of *Artemia franciscana*. The authors observed low bioaccumulation of metals alone by *A. franciscana* when compared with similar data from the literature, but also stated the occurrence of synergistic effects when animals were simultaneously exposed to organotins and heavy metals.

Species and strain differences were studied by Sarabia et al. (2002) who used several species and strains of the genus *Artemia* as test organisms for the assessment of cadmium toxicity. The species and strains were selected according to distinct patterns of reproduction, ploidy and site of origin. Marked differences were observed after acute exposures, which confirms again the need for a detailed characterization of the specimens used in toxicity assays. These authors also implemented a sensitivity ranking, in which the most sensitive species tested was *A. franciscana*, while *A. persimilis* was the most resistant; nevertheless, *Artemia* species were considered as being among the most insensitive crustaceans to cadmium toxic effects. This ranking allowed several interpretations of sensitivity/resistance according to certain parameters, such as habitat peculiarities and historic origin of the populations. Highly consistent mortality data within the same species after cadmium exposure strongly suggests a major genetic influence on the mechanism of toxicity. Parthenogenetic *Artemia* evidenced a reduced toxicity after cadmium exposure, which is in agreement with the hypothesis stating that parthenogenetic populations are widely distributed due to their high overall resistance to stressing environmental factors.

Besides species- and strain-specific differences, several other factors contribute to the variability in results obtained when using *Artemia* nauplii. One of the major influencing factors is the age of the organism used. Barahona and Sánchez-Fortún (1996) reported on the exposure of three different life stages of *A. salina* to several phenolic compounds; their conclusions indicate that sensitivity to the selected chemicals increased as the test organism developed. Increased age resulted in increased toxicity expression, at least when comparing LC_{50} values obtained when using specimens with ages comprised between 24 and 48 h. The use of 168 h-old nauplii, did not result in enhanced toxicity expression at least for the majority of the tested compounds. This study allowed to compare the most suitable age class for toxicological testing (48 h for *Artemia*) with other well-established aquatic animal models, such as *Daphnia magna*, which possessed an analogous response between aging and toxicity expression/enhanced sensitivity. These data show that animals of clearly defined age classes must be used for toxicity assays, since the toxicity expression is directly influenced by the development stage of the test organisms.

Parra et al. (2001) assessed the effect of acute treatment of *Artemia salina* larvae and mice with several extracts drawn from autochthonous plants of Cuba. This study intended to develop a low cost methodology applicable to countries where the use of medicines obtained from vegetable species is common and is an affordable manner of fighting disease. The authors calculated LC_{50} values for *Artemia salina* larvae and LD_{50} values for mice and established significant correlations between both parameters, suggesting the use of *Artemia salina* larvae as a suitable, accurate and inexpensive alternative to pre-screening chemical toxicity with mammals. This study was in accordance with the earlier work from Guilhermino et al. (2000), where a correlation between *Daphnia magna* LC_{50} values and rat LD_{50} for 15 compounds was established. This research assumes particular relevance since regulatory aspects of the introduction of new methodologies and testing protocols continues to be an emergent priority for international institutions involved in the definition and validation of toxicity testing and assessment guidelines. Nevertheless, both papers underline the importance of validating assays with invertebrates as pre-screening methods, reducing the need for bioassays with mammals.

van Wezel et al. (2000) referred another potential application of *Artemia*-derived assays for the establishment of Environmental Risk Limits (ERLs) for two phthalates. Due to the availability of toxicity data concerning phthalates for a group of several distinct species, in which *Artemia salina* was included, the authors were able to define numerical values of concentrations that can eventual lead to the exertion of toxic effects over several elements of the studied ecosystems. The authors state that the attainment of such values is of superior importance in order to assure a suitable level of protection against chemical insults caused by the environmental exposure to this type of compounds.

7. Conclusions

Artemia is one of the most valuable test organisms available for ecotoxicity testing and research done so far allows us to state that it is possible to sustain several options related to *Artemia* use in Toxicology and Ecotoxicology. As it was highlighted in this review article, adaptability of *Artemia* genus to distinct environmental conditions may turn *Artemia*, in the future, into a crucial test organism for Ecotoxicology testing. Its natural tolerance may be faced as an advantage, in comparison to other test organisms, less adapted to a large number of abiotic conditions.

Artemia-based testing can involve cyst-based assays, or it can depend on animals cultured in the laboratory. Cyst-based toxicity assays are cheap, continuously available, simple and reliable and are thus an important answer to routine needs of toxicity screening, for industrial monitoring requirements or for regulatory purposes. Cultured animals (clones) can be more suited to face the needs of basic scientific research, due to their intrinsic low genetic variability and consequent homogeneity. Laboratory rearing of several species of *Artemia* is simple and inexpensive and is favoured by its flexibility for nutrient

sources, temperature and salinity tolerance, easy manipulation, short generation times and high reproductive capacity.

The targeted end-points depend on the phase of the life cycle of the test organisms used, and can thus differ from hatchability of cysts to mortality of nauplii. Responses drawn from ecotoxicity tests might not only be ''binary'' answers (life vs. death) but also enzymological (e.g. enzymatic biomarkers), physiological (e.g. oxygen consumption rates) or reproductive (e.g. reproductive output).

Artemia can be used alone, as a single test species in toxicity screening and mechanistic toxicological studies, or combined into broader studies, simultaneously with several other species. This is a helpful approach for holistic interpretations of risks related to global ecosystems, through the integration of varied responses, at various organizational and trophic levels.

Artemia can be subjected to both field and laboratory testing conditions. Tolerance of *Artemia* specimens makes this genus adaptable to a great variety of testing conditions, in estuarine, marine or hypersaline environments, thus responding to the actual demands of standardized tests for saline ecosystems.

Genetic variability among parthenogenetic strains can be transformed into an advantage, through selection of the most suitable strain, complying with requirements of sensitivity, reproductive features, life history traits, relative abundance and geographical distribution.

Acknowledgements

The present work was partially funded by ''Fundação para a Ciência e a Tecnologia'' (B. Nunes Ph.D. grant SFRH/BD/866/2000) and by Project ''CONTROL'' (POCTI/MAR/MAR/15266/1999).

References

Abatzopoulos, T.J., Triantaphyllidis, G.V., Beardmore, J.A., Sorgellos, P., 1997. Cyst Membrane Composition as a Discriminant Character in the Genus *Artemia*. (International Study on *Artemia* LV). Journal of the Marine Biology Association of the United Kingdom 77, 265–268.

Amat, F., 1985. Biologia de *Artemia*. Informes Técnicos del Instituto de Investigaciones Pesqueras.

Barahona, M.V., Sánchez-Fortún, S., 1996. Comparative Sensitivity of Three Age Classes of *Artemia salina* Larvae to Several Phenolic Compounds. Bulletin of Environmental Contamination and Toxicology 56, 271–278.

Barahona, M.V., Sánchez-Fortún, S., 1998. Toxicity of Carbamates to the Brine Shrimp *Artemia salina* and the Effect of Atropine, Iso-ompa and 2-PAM on carbaryl toxicity. Environmental Pollution 104, 469–476.

Barahona-Gomariz, M.V., Sanz-Barrera, F., Sánchez-Fortún, S., 1994. Acute Toxicity of Organic Solvents on *Artemia salina*. Bulletin of Environmental Contamination and Toxicology 52, 766–771.

Barata, C., Hontoria, F., Amat, F., Browne, R., 1996. Competition between sexual and parthenogenetic *Artemia*: temperature and strain effects. Journal of Experimental Marine Biology and Ecology 196, 313–328.

Beattie, K.A., Ressler, J., Wiegand, C., Krause, E., Codd, G.A., Steinberg, C.E.W., Pflugmacher, S., 2003. Comparative effects and metabolism of two microcystins and nodularin in the brine shrimp *Artemia salina*. Aquatic Toxicology 62, 219–226.

Blaise, C., 1998. Microbiotesting: An Expanding Field in Aquatic Toxicology. Ecotoxicology and Environmental Safety 40, 115–119.

Bossier, P., Xiaomei, W., Catania, F., Dooms, S., Van Stappen, G., Naessens, E., Sorgeloos, P., 2004. An RFLP database for authentication of commercial cyst samples of the brine shrimp *Artemia* spp. (International Study on *Artemia* LXX). Aquaculture 231, 93–112.

Bowen, S.T., Fogarino, E.A., Hitchner, K.N., Dana, G.L., Chow, V.H.S., Buonchristiani, M.R., Carl, J.R., 1985. Ecological isolation in *Artemia*: population differences in tolerance of anion concentrations. Journal of Crustacean Biology 5, 106–129.

Brix, K.V., Cardwell, R.D., Adams, W.J., 2003. Chronic toxicity of arsenic to the Great Salt Lake brine shrimp, *Artemia franciscana*. Ecotoxicology and Environmental Safety 54, 169–175.

Browne, R.A., 1980. Acute response versus reproductive performance in five strains of brine shrimp exposed to copper sulphate. Marine Environmental Research 3, 185–193.

Browne, R.A., 1992. Population Genetics and Ecology of *Artemia*: Insights into Parthenogenetic Reproduction. Trends in Ecology and Evolution 7, 232–237.

Browne, R.A., Halanych, K.M., 1989. Competition between sexual and parthenogenetic *Artemia*: a re-evaluation (Branchiopoda, Anostraca). Crustaceana 57, 57–71.

Browne, R.A., Wanigasekera, G., 2000. Combined effects of salinity and temperature on survival and reproduction of five species of *Artemia*. Journal of Experimental Marine Biology and Ecology 244, 29–44.

Browne, R.A., Davis, L.E., Sallee, S.E., 1988. Effects of temperature and relative fitness of sexual and asexual brine shrimp *Artemia*. Journal of Experimental Marine Biology and Ecology 124, 1–20.

Browne, R.A., Moller, V., Forbes, V.E., Depledge, M.H., 2002. Estimating genetic and environmental components of variance using sexual and clonal *Artemia*. Journal of Experimental Marine Biology and Ecology 267, 107–119.

Bruggeman, E., Sorgeloos, P., Vanhaecke, P., 1980. Improvements in the decapsulation technique of *Artemia* cysts. In: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), The Brine Shrimp Artemia. Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, pp. 261–269.

Calleja, M.C., Geladi, P., Persoone, G., 1994. Modelling of human acute toxicity from physicochemical properties and non-vertebrate acute toxicity of the 38 organic chemicals of the MEIC priority list by PLS regression and neural network. Food and Chemical Toxicology 32, 923–941.

Carballo, J.L., Hernández-Inda, Z.L., Pérez, P., García-Grávalos, M.D., 2002. A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. BMC Biotechnology 2, 17.

Clegg, J.S., 2001. Cryptobiosis-a peculiar state of biological organization. Comparative Biochemistry and Physiology Part B 128, 613–624.

Creasel, 1990. ARTOXKIT M: *Artemia* Toxicity Screening Test for Estuarine and Marine Waters. Standard Operational Procedure V071090.

Crisinel, A., Delaunay, L., Rossel, D., Tarradellas, J., Meyer, H., Saiah, H., Vogel, P., Delisle, C., Blaise, C., Hansen, P.D., 1994. Cyst-based ecotoxicity tests using Anostracans: Comparison of two species of Streptocephalus. Environmental Toxicology and Water Quality 9, 317.

Di Delupis, G.D., Rotondo, V., 1988. Phototaxis in aquatic invertebrates: possible use in ecotoxicity tests. Ecotoxicology and Environmental Safety 16, 189–193.

Dobbeleir, J., Adam, N., Bossuyt, E., Bruggeman, E., Sorgeloos, P., 1980. New aspects of the use of inert diets for high density culturing of brine shrimp. The Brine Shrimp Artemia. In: Ecology, Culturing, Use in Aquaculture, vol. 3, pp. 165–174.

Dhont, J., Sorgeloos, P., 2002. Applications of *Artemia*. In: Abatzopoulos, T.J., Beardmore, J.A., Clegg, J.S., Sorgeloos, P. (Eds.), *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 251–277.

Dhont, J., Van Stappen, G., 2003. Biology, Tank Production and Nutritional Value of *Artemia*. In: Støttrup, J.G., McEvoy, L.A. (Eds.), Live Feeds in Marine Aquaculture. Blackwell Publishing Oxford, UK, pp. 65–121.

Evjemo, J.O., Olsen, Y., 1999. Effect of food concentration on the growth and production rate of *Artemia franciscana* feeding on algae (*T. iso*). Journal of Experimental Marine Biology and Ecology 242, 273–296.

Gaggi, C., Sbrilli, G., Hasab El Naby, A.M., Bucci, M., Duccini, M., Bacci, E., 1994. Toxicity and Hazard Ranking of s-Triazine Herbicides Using

Microtox®, Two Green Algal Species and a Marine Crustacean. Environmental Toxicology and Chemistry 14, 1065–1069.

Gajardo, G., Abatzopoulos, T.J., Kappas, I., Beardmore, J.A., 2002. Evolution and Speciation. In: Abatzopoulos, T.J., Beardmore, J.A., Clegg, J.S., Sorgeloos, P. (Eds.), *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 225–250. (*Chapter V*).

Guerra, R., 2001. Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents. Chemosphere 44, 1737–1747.

Guilhermino, L., Diamantino, T., Silva, M.C., Soares, A.M.V.M., 2000. Acute Toxicity Test with *Daphnia magna*: An Alternative to Mammals in the Prescreening of Chemical Toxicity? Ecotoxicology and Environmental Safety 46, 357–362.

Hadjispyrou, S., Kungolos, A., Anagnostopoulos, A., 2001. Toxicity, Bioaccumulation, and Interactive Effects of Organotin, Cadmium, and Chromium on *Artemia franciscana*. Ecotoxicology and Environmental Safety 49, 179–186.

Hlywka, J.J., Beck, M.M., Bullerman, L.B., 1997. The use of the Chicken Embryo Screening Test and Brine Shrimp (*Artemia salina*) Bioassays to assess the toxicity of Fumonisin B_1 Mycotoxin. Food and Chemical Toxicology 35, 991–999.

Kapanen, A., Itävaara, M., 2001. Ecotoxicity Tests for Compost Applications. Ecotoxicology and Environmental Safety 49, 1–16.

Knulst, J., Sodergren, A., 1994. Occurrence and Toxicity of Persistent Pollutants in Surface Microlayers Near an Incineration Plant. Chemosphere 29, 1339–1347.

Lavens, P., Sorgeloos, P., 1987. The cryptobiotic state of *Artemia* cysts, its diapause deactivation and hatching: a review. In: Sorgeloos, P., Bengtson, D.A., Decleir, W., Jaspers, E. (Eds.), *Artemia* Research and its Applications. Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, pp. 27–63. (556).

Lavens, P., Sorgeloos, P.,, 1996. Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper No. 361, pp. 295.

Lavens, P., Sorgeloos, P., 2000. The history, present status and prospects of the availability of *Artemia* cysts for aquaculture. Aquaculture 181, 397–403.

Léger, P., Bengtson, D.A., Simpson, K.L., Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source. Oceanography and Marine Biology: An Annual Review 24, 521–623.

Léger, P., Bengtson, D.A., Sorgeloos, P., Simpson, K.L., Beck, A.D., 1987a. The nutritional value of *Artemia*: a review. In: Sorgeloos, P., Bengtson, D.A., Decleir, W., Jaspers, E. (Eds.), *Artemia* Research and its Applications. Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, pp. 357–372.

Léger, P.H., Naessens-Foucquaert, E., Sorgeloos, P., 1987b. International Study on *Artemia*. XXXV. Techniques to manipulate the fatty acid profile in *Artemia* nauplii and the effect on its nutritional effectiveness for the marine crustacean *Mysidopsis bahia* (M.). In: Sorgeloos, P., Bengtson, D.A., Decleir, W., Jaspers, E. (Eds.), Artemia Research and its Applications. Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, pp. 411–424.

Migliore, L., Dojmi di Delupis, G., Capellaro, H., Brambilla, G., 1993. Drugs in Aquaculture: Monitoring of Contamination and proposal for its reduction by the use of *Artemia salina* (L.) in Lakes and Reservoirs. In: Giusani, G., Callieri, C. (Eds.), Strategies for Lake Ecosystems Beyond 2000. Griggi G.M., Baveno, Italy, pp. 291–294.

Migliore, L., Civitareale, C., Brambilla, G., Dojmi di Delupis, G., 1997. Toxicity of Several Important Agricultural Antibiotics to *Artemia*. Water Research 31, 1801–1806.

Naegel, L.C.A., 1999. Controlled production of *Artemia* biomass using an inert commercial diet compared with the microalgae *Chaetoceros*. Aquacultural Engineering 21, 49–59.

Nałęcz-Jawecki, G., Grabińska-Sota, E., Narkiewicz, P., 2003. The toxicity of cationic surfactants in four bioassays. Ecotoxicology and Environmental Safety 54, 87–91.

Nascimento, A., Smith, D.H., Pereira, S.A., Sampaio de Araújo, M.M., Silva, M.A., Mariani, A.M., 2000. Integration of varying responses of different organisms to water and sediment quality at sites impacted and not impacted by the petroleum industry. Aquatic Ecosystem Health and Management 3, 449–458.

Nipper, M., 2000. Current approaches and future directions for contaminant-related impact assessments in coastal environments: Brazilian perspective. Aquatic Ecosystem Health and Management 3, 433–447.

Okamura, H., Aoyama, I., Liu, D., Maguire, R.J., Pacepavicius, G.J., Lau, Y.L., 2000. Fate and Ecotoxicity of the New Antifouling Compound Irgarol 1051 in the Aquatic Environment. Water Research 34, 3523–3530.

Oliveira-Filho, E.C., Paumgartten, F.J.R., 2000. Toxicity of *Euphorbia milii* Latex and Niclosamide to Snails and Nontarget Aquatic Species. Ecotoxicology and Environmental Safety 46, 342–350.

Panagoula, B., Panayiota, M., Iliopoulou-Georgudaki, J., 2002. Acute Toxicity of TBT and IRGAROL in *Artemia salina*. International Journal of Toxicology 21, 231–233.

Panigrahi, S., 1993. Bioassay of mycotoxins using terrestrial and aquatic, animal and plant species. Food Chemistry and Toxicology 31, 767–790.

Parra, L.A., Silva Yhebra, R., Guerra Sardiñas, I., Iglesias Buela, L., 2001. Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD_{50} value) in mice, to determine oral acute toxicity of plant extracts. Phytomedicine 8 (5), 395–400.

Persoone, G., Vanhaecke, P.,, 1981. Intercalibration exercise on a short-term standard toxicity test with Artemia naplii. Final report. Contract CEE-ENV-396 B(N), 30.

Persoone, G., Wells, P.G., 1987. *Artemia* in aquatic toxicology: A review. In: Sorgellos, P., Bengtson, D.A., Decleir, W., Jaspers, E. (Eds.), *Artemia* Research and its Applications. Morphology, Genetics, Strain Characterization, Toxicology, vol. I. Universa Press, Wetteren, Belgium.

Persoone, G., Blaise, C., Snell, T., Janssen, C., Van Steertegem, M., 1992. Cyst-based toxicity tests: II.– Report on an international intercalibration exercise with three cost-effective Toxkits. Zeitschrift für Angewandte Zoologie 17–36.

Petrucci, F., Caimi, S., Mura, G., Caroli, S., 1995. *Artemia* as a Test organism of Environmental Contamination by Trace Elements. Microchemical Journal 51, 181–186.

Sánchez-Fortún, S., Sanz, F., Barahona, M.V., 1996. Acute Toxicity of Organophosphorous Insecticides and Protection by Cholinergic Antagonists and 2-PAM on *Artemia salina* larvae. Archives of Environmental Contamination and Toxicology 31, 391–398.

Sarabia, R., Varó, I., Torreblanca, A., del Ramo, J.J., Pastor, A., Amat, F., Díaz-Mayans, J., 1998a. Accumulation of Cadmium in Several Strains of *Artemia*. Cuadernos de Investigación Biológica 20, 435–438.

Sarabia, R., Torreblanca, A., Del Ramo, J.J., Díaz-Mayans, J., 1998b. Effects of low mercury concentration exposure on hatching, growth and survival in the *Artemia* strain La Mata parthenogenetic diploid. Comparative Biochemistry and Physiology Part A 120, 93–97.

Sarabia, R., Del Ramo, J., Varó, I., Díaz-Mayans, J., Torreblanca, A., 2002. Comparing the Acute Exposure to Cadmium Toxicity of Nauplii from Different Populations of *Artemia*. Environmental Toxicology and Chemistry 21, 437–444.

Soares, A.M.V.M., Baird, D.J., Calow, P., 1992. Interclonal Variation in the Performance of *Daphnia magna* Straus in Chronic Bioassays. Environmental Toxicology and Chemistry 11, 1477–1483.

Song, M.Y., Brown, J.J., 1998. Osmotic Effects as a Factor Modifying Insecticide Toxicity on *Aedes* and *Artemia*. Ecotoxicology and Environmental Safety 41, 195–202.

Sorgeloos, P., 1980. Availability of reference *Artemia* cysts. Marine Ecology Progress Series 3, 363–364.

Sorgeloos, P., Baeza-Mesa, M., Bossuyt, E., Bruggeman, E., Dobbeleir, J., Versichele, D., Laviña, E., Bernardino, A., 1980. Culture of *Artemia* on rice bran: the conversion of a waste-product into highly nutritive animal protein. Aquaculture 21, 393–396.

Sorgeloos, P., Coutteau, P., Dhert, P., Merchie, G., Lavens, P., 1998. Use of the brine shrimp, *Artemia* spp., in larval crustacean nutrition: a review. Review in Fisheries Science 6, 55–68.

Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147–159.

Tong, Z., Hongjun, J., Huailan, Z., 1996. Quality criteria of acrylonitrile for the protection of aquatic life in China. Chemosphere 32, 2083–2093.

Tothill, I.E., Turner, A.P.F., 1996. Developments in bioassay methods for toxicity testing in water treatment. Trends in Analytical Chemistry 75, 178–188.

Touraki, M., Niopas, I., Kastritsis, C., 1999. Bioaccumulation of Trimethoprim, Sulfamethoxazole and *N*-acetyl-sulfamethoxazole in *Artemia* nauplii and residual kinetics in seabass larvae after repeated oral dosing of medicated nauplii. Aquaculture 175, 15–30.

Triantaphyllidis, G.V., Pilla, E.J.S., Thomas, K.M., Abatzopoulos, T.J., Beardmore, J.A., Sorgeloos, P., 1994. International Study on *Artemia*.52. Incubation of *Artemia* Cyst Samples at High Temperatures Reveals Mixed Nature with *Artemia franciscana* Cysts. Journal of Experimental Marine Biology and Ecology 183, 273–282.

Triantaphyllidis, G.V., Poulopoulou, K., Abatzopoulos, T.J., Pinto Perez, C.A., Sorgeloos, P., 1995. International study on *Artemia*. XLIX. Salinity effects on survival, maturity, growth, biometrics, reproductive and lifespan characteristics of a bisexual and a parthenogenetic population of *Artemia*. Hydrobiologia 302, 215–227.

Triantaphyllidis, G.V., Abatzopoulos, T.J., Sorgeloos, P., 1998. Review of the biogeography of the genus *Artemia* (Crustacea, Anostraca). Journal of Biogeography 25, 213–226.

Van Stappen, G., 2002. Zoogeography: In: Abatzopoulos, Th. J., et al. (Eds.), Artemia: Basic and Applied Biology. Kluwer Academic Publishers. 171–224.

Van Stappen, G., 2003. Production, Harvest and Processing of *Artemia* from Natural Lakes. In: Støttrup, J.G., McEvoy, L.A. (Eds.), Live Feeds in Marine Aquaculture. Blackwell Publishing Oxford, UK, pp. 122–144.

Vanhaecke, P., Persoone, G., 1981. Report on an intercalibration exercise on a short-term standard toxicity test with *Artemia* nauplii (ARC-test). Institut National de la Santé et de la Recherche Médicale (INSERM) 106, 359–376.

Vanhaecke, P., Sorgeloos, P., 1980a. International study on *Artemia*: 4. The biometrics of *Artemia* strains from different geographical origin. The Brine Shrimp *Artemia*. In: Ecology, Culturing, Use in Aquaculture, vol. 2, pp. 393–405.

Vanhaecke, P., Sorgeloos, P., 1980b. International study on *Artemia*: 14. Growth and survival of *Artemia* larvae of different geographical origin in a standard culture test. Marine Ecology Progress Series 3, 303–307.

Vanhaecke, P., Sorgeloos, P., 1982. International study on *Artemia*: 18. The hatching rate of *Artemia* cysts: a comparative study. Aquaculture Engineering 1, 263–273.

Vanhaecke, P., Sorgeloos, P., 1983. International study on *Artemia*: 19. Hatching data for ten commercial sources of brine shrimp cysts and re-evaluation of the "hatching efficiency" concept. Aquaculture 30, 43–52.

Vanhaecke, P., Sorgeloos, P., 1989. International Study on *Artemia*. XLVII. The effect of temperature on cyst hatching, larval survival and biomass production for different geographical strains of brine shrimp *Artemia* spp. Annales de la Société royale zoologique de Belgique 119, 7–23.

Vanhaecke, P., Steyaert, H., Sorgeloos, P., 1980a. International study on *Artemia*: 3. The use of Coulter Counter® equipment for the biometrical analysis of *Artemia* cysts: methodology and mathematics. The Brine Shrimp Artemia. In: Ecology, Culturing, Use in Aquaculture, vol. 1, pp. 107–115.

Vanhaecke, P., Persoone, G., Claus, C., Sorgeloos, P., 1980b. Research on the development of a short-term standard toxicity test with *Artemia*. The Brine Shrimp Artemia. In: Ecology, Culturing, Use in Aquaculture, vol. 1, pp. 263–285.

Vanhaecke, P., Cooreman, A., Sorgeloos, P., 1981a. International study on *Artemia*: 15. Effect of light intensity on hatching rate of *Artemia* cysts from different geographical origin. Marine Ecology Progress Series 5, 111–114.

Vanhaecke, P., Persoone, G., Claus, C., Sorgeloos, P., 1981b. Proposal for a short-term toxicity test with *Artemia* nauplii. Ecotoxicology and Environmental Safety 5, 328–387.

Vanhaecke, P., Siddal, S.E., Sorgeloos, P., 1984. International Study on *Artemia*. XXXII. Combined effects of temperature and salinity on the survival of *Artemia* of various geographical origin. Journal of Experimental Marine Biology and Ecology 80, 259–275.

Varó, I., Taylor, A.C., Ferrando, M.D., Amat, F., 1997. Effect of Endosulfan Pesticide on the Oxygen Consumption Rates of Nauplii of Different Spanish Strains of *Artemia*. Journal of Environment Science and Health–Part B: Pesticides, Food Contaminants and Agricultural Wastes 32, 363–375.

Varó, I., Serrano, R., Navarro, J.C., López, F.J., Amat, F., 1998. Acute Lethal Toxicity of the Organophosphorous Chlorpyrifos to Different Species and Strains of *Artemia*. Bulletin of Environmental Contamination and Toxicology 61, 778–785.

Varó, I., Serrano, R., Pitarch, E., Amat, F., López, F.J., Navarro, J.C., 2000. Toxicity and Bioconcentration of Chlorpyrifos in Aquatic Organisms: *Artemia parthenogenetica* (Crustacea), *Gambusia affinis*, and *Aphanius iberus* (Pisces). Bulletin of Environmental Contamination and Toxicology 65, 623–630.

Varó, I., Navarro, J.C., Amat, F., Guilhermino, L., 2002a. Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. Chemosphere 48, 563–569.

Varó, I., Serrano, R., Pitarch, E., Amat, F., Lopez, F.J., Navarro, J.C., 2002b. Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish. Archives of Environmental Contamination and Toxicology 42, 229–235.

Vezie, C., Sivonen, K., Brient, L., Bertru, G., Lefeuvre, J.C., 1996. Development of Toxic Cyanobacteria in Western France-Detection of Toxicity with *Artemia salina* Tests. Annales de Limnologie 32, 123–128.

Vismara, C., 1998. Effects of methanol, ethanol and *n*-propanol on development of *Artemia salina* cysts. Chemosphere 37 (14–15), 3027–3034.

Wells, P.G., 1999. Biomonitoring the Health of Coastal Marine Ecosystems: The Roles and Challenges of Microscale Toxicity Tests. Marine Pollution Bulletin 39, 39–47.

van Wezel, A.P., van Vlaardingen, P., Posthumus, R., Crommentuijn, G.H., Sijm, D.T.H.M., 2000. Environmental Risk Limits for Two Phthalates, with Special Emphasis on Endocrine Disruptive Properties. Ecotoxicology and Environmental Safety 46, 305–321.

Zmora, O., Avital, E., Gordin, H., 2002. Results of an attempt for mass production of *Artemia* in extensive ponds. Aquaculture 213, 395–400.